USING IONIC STRENGTH TO MODULATE LIPID DIFFUSION IN SOLID-SUPPORTED LIPID BILAYERS

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ABSTRACT

Solid-supported lipid bilayers (SLBs) are an ideal model system to study natural cell membranes. They can be used as a medium for protein separation, a target for virus fusion, and as an integral component of biosensors. These applications often require that the bilayer contain zwitterionic (neutral) and negatively charged lipids in an environment of non-zero ionic strength, as is the case with natural cell membranes. In this work the effect of ionic strength on the fluidity of mixed zwitterionic/anionic solid-supported lipid bilayers is investigated using fluorescence recovery after photobleaching (FRAP). Ionic strength of up to 3000 mM was found to have very little impact on the fluidity of purely zwitterionic bilayers. In the case of the mixed zwitterionic/anionic bilayers however, it was found that even small levels of ionic strength significantly reduced the diffusion coefficient of the anionic lipids. These results were supported by light scattering measurements of vesicles that indicate ions have strong interactions with anionic lipids, but not zwitterionic ones. This work provides insight into the behavior of lipids in natural cell membranes and underscores the importance of appropriate fluorescent probe selection in diffusion measurement studies. Additionally, it demonstrates a technique for controlling the fluidity of lipid bilayers by simply adjusting the ionic strength of the buffer surrounding the sample as opposed to altering the composition or substrate of the bilayer. This technique allows one to investigate the effect of diffusion within one specific sample, eliminating the effect of batch to batch variability in sample preparation.

BIOGRAPHICAL SKETCH

Sudhir Prabhu obtained his B.S. in chemical engineering from Johns Hopkins University in 2005 with a minor in Entrepreneurship and Management. He then worked as a biochemical engineer in the Department of Fermentation and Cell Culture Process Development at Merck Inc. in West Point, Pennsylvania from 2005-2007. Here his focus was on optimizing mammalian cell cultures for the production of therapeutic proteins. In the fall of 2007 he began his graduate studies at Cornell University under the supervision of Professor Susan Daniel.

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CHAPTER 1.

INTRODUCTION AND BACKGROUND

Conflicting experimental and computational reports of the effect of ionic strength on the fluidity of zwitterionic multilamellar lipid bilayers have been published. Some authors argue that small levels of ionic strength have a large impact on the fluidity of these bilayers due to ions binding multiple lipids together into larger slow moving complexes (Bockmann 2003, Gurtovenko 2008). Others have argued that an impact of ionic strength on zwitterionic bilayer fluidity only occurs at high ionic strengths above 1000 mM due to the electrostatic screening enabling denser packing of lipids (Pabst 2007, Kotulska 2005, Fillipov 2009).

In this work, the effect of ionic strength on the mobility of zwitterionic and mixed 99.9% zwitterionic/0.1% anionic solid-supported unilamellar lipid bilayers is investigated using fluorescence recovery after photobleaching (FRAP). Ionic strengths of up to 3000 mM was found to have very little impact on the fluidity of purely zwitterionic bilayers. In the case of the mixed zwitterionic/anionic bilayers however, it was found that even small levels of ionic strength significantly reduced the diffusion coefficient of the anionic lipids, with the divalent salt $CaCl_2$ having a stronger impact than the monovalent salt NaCl. This effect of ionic strength on the diffusion of anionic lipids was found to be completely reversible for both salts. The FRAP results were supported by light scattering measurements of vesicles that indicate ions have strong (yet reversible) interactions with anionic lipids, but not zwitterionic ones. These results provide insight into selecting the appropriate fluorescently labeled lipid for FRAP experiments, demonstrate a technique for controlling the fluidity of lipid bilayers, and perhaps provide some insight into how the cell might locally control diffusion in its membrane.

Preliminary experiments investigating the effect of ionic strength on mixed 99.9% zwitterionic/0.1% anionic and 75% zwitterionic/25% anionic bilayers formed on a positively-charged polyelectrolyte layer were also conducted. The use of a positively-charged polyelectrolyte layer allows for the formation of lipid bilayers supported on glass with high levels of anionic lipids.

1.1 Lipids

Cell membranes are composed mostly of lipids and proteins. The three major types of lipids in a cell membrane are phospholipids, glycolipids, and cholesterol. In this work, phospholipids are studied. These membrane lipids are amphipathic molecules having a hydrophilic (polar) head group and a pair of hydrophobic acyl chain tails. Because of this ampipathic nature, in water these lipids will self-assemble into spherical vesicles or in the presence of a solid support such as glass, a bimolecular sheet closely adsorbed to the support. One of the most abundant lipids present in natural cell membranes is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Figure 1). This lipid is zwitterionic, possessing a head group with both a positive and negative charge. The net charge however, is neutral.

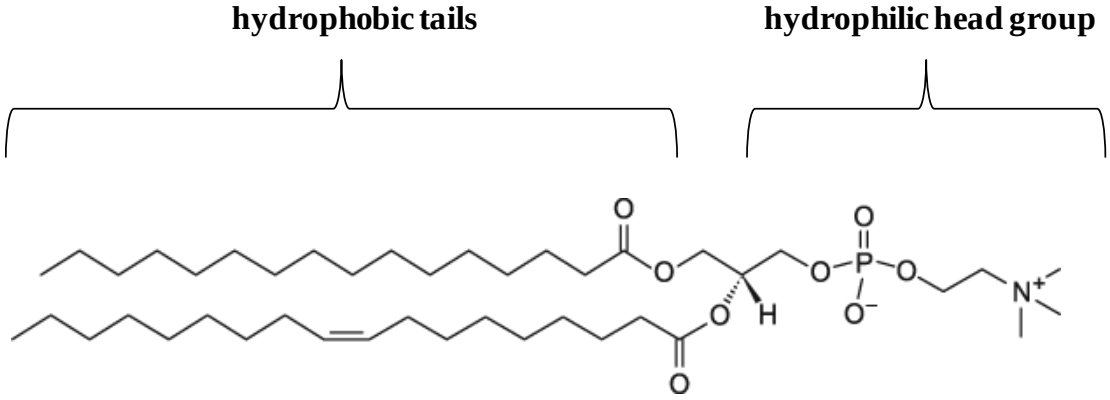


Figure 1: Structure of the zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid (Avanti Polar Lipids, Inc.).

Another common anionic lipid found in natural cell membranes is 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) (Figure 2). This lipid is typically found on the cytosolic side of the cell membrane.

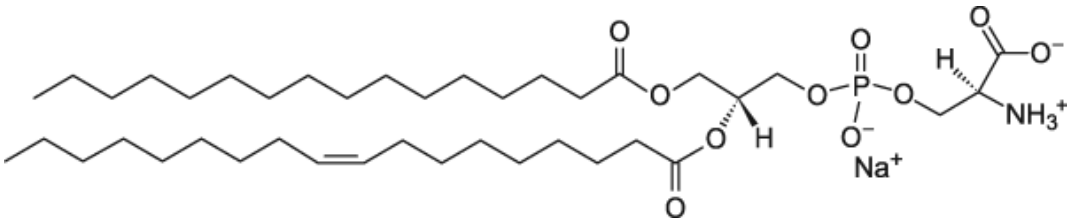


Figure 2: Structure of the anionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) lipid (Avanti Polar Lipids, Inc.).

1.2 Solid-supported lipid bilayers

Commercially available lipids in chloroform solutions are used to make solid-supported lipid bilayers. The volatile organic solvent is removed by drying, and the dried lipids are then resuspended in buffer. The resulting multilamellar vesicles are subjected to several freeze/thaw cycles and then passed through an extruder. The freeze/thaw cycles transform the multilamellar vesicles into unilamellar ones which are only one bilayer thick. The extruder reduces the size of these unilamellar vesicles to sizes that are not thermodynamically stable (~100 nm). These small unilamellar lipid vesicles rupture upon contact with a solid support such as quartz or glass to form a unilamellar lipid bilayer sheet adsorbed near the glass surface. The formation of the bilayer is governed by Van der Waals, electrostatic, and hydration forces (Israelachvili 1992). Excess vesicles are removed by vigorous buffer exchange. Lipid bilayers will form on untreated glass, but chemical treatments such as piranha solution result in bilayers with higher levels of fluidity. Piranha solution is a mixture of sulfuric acid and hydrogen peroxide that is a strong oxidizer. It will hydroxylate the surface of glass by increasing silanol groups and Si-O species which make the glass more hydrophilic (Seu 2007). The lipid bilayers that form on these surfaces are

approximately 4 nm thick and are separated from the solid support by a 1 nm thick water layer (Koenig 1996, Bayerl 1990, Johnson 1991).

There are many benefits of using SLBs as a model to study natural cell membranes. While natural cell membranes are very complex and contain many species, the composition and environment of solid-supported lipid bilayers can be precisely controlled. This enables one to more easily identify the effects of certain parameters (ionic strength, pH, etc.) on an individual component. This is especially true since the two-dimensional planar nature of SLBs make them very amenable to surface analysis using techniques such as total internal reflection fluorescence microscopy (Oreopoulos 2009), atomic force microscopy (Richter 2006), surface plasmon resonance (Lahiri 2000), and ellipsometry (Dubas 2001).

Additionally, the components in solid-supported lipid bilayers are mobile, as they are in natural cell membranes. This mobility is necessary for many naturally occurring processes such as multivalent ligand-receptor binding (Deng 2008), protein-protein interactions (Vaz 1984), and assembly of signaling rafts (Milhiet 2001).To date, most research on the effect of ionic strength on lipid bilayers has been done using multilamellar bilayer systems (Bockmann 2003, Fillipov 2009,
Pabst 2007). These bilayers are formed by depositing lipids in a volatile organic solvent on a glass coverslip. The solvent is removed by drying and then replaced with water. This results in the self-assembly of multiple bilayers stacked on top of one another. This system of multilayers does not represent in vivo conditions, where the plasma membrane is one layer thick, exposed to aqueous solutions on both sides can interact with the cell's cytoskeleton. Additionally, it is well known that salt addition causes "swelling" in multilamellar systems. This "swelling" is thought to occur because ion association with lipids leads to electrostatic repulsion between bilayers (Petrache 2006). Because of these drawbacks, solid-supported lipid bilayers are more

similar to physiological systems and are better suited to studying the effect of ionic strength on lipid bilayers.

1.3 Diffusion of lipids in solid-supported lipid bilayers

Many theoretical models have been published describing the diffusion of molecules in lipid bilayers. For diffusants similar in size to the "solvent" (i.e. a POPS lipid diffusing in a POPC bilayer), a free area or lattice model such as the following is appropriate (Hallman 1985):

$$D = \left(\frac{kT}{f}\right) exp\left[\frac{-\gamma a^*}{a_0[\beta+\alpha_a(T-T_m)]}\right] \quad (1)$$

where D is the translational diffusion of the lipid, k is Boltzmann's constant, T is the temperature, *f* is the translational friction coefficient, T_m is the transition temperature of the lipid bilayer, γ is a numerical factor that accounts for overlap of free area, a* is the critical free area (the minimum area necessary for lipid translation), a_0 is the van der Waals area of the lipid, $a_0\beta$ is the area at the T_m, and α_a is the lateral thermal expansion coefficient of the bilayer. Unfortunately, many of these constants for a given system, especially a*, are difficult to obtain. For this reason, many have used a continuum fluid hydrodynamic model, the Saffman-Delbruck equation, to analyze diffusion in lipid bilayers. Although this equation is based on the assumption that the diffusant is much larger than the "solvent" (i.e. a protein diffusing in a POPC bilayer), many authors have utilized it for systems where this assumption does not hold and shown that its predictions are in line with experimental measurements (Kuhner 1994, Evans 1988). The following equation is a modified version of the Saffman-Delbruck equation that takes into account the coupling of a lipid bilayer to a substrate though a thin lubricating layer as in a SLB (Kuhner 1994):

$$D = \frac{kTh}{\pi \eta_w a_p^2} \quad (2)$$

where D is the diffusion coefficient of the lipid, T is the temperature, *h* is the height of the confined water layer, η_w is the viscosity of the confined water layer, and a_p^2 is the van der Waals radius of the lipid. Using this equation it was shown that the predicted diffusion coefficient of NBD lipids matched quite closely to the experimental values (Kuhner 1994). It can be seen from this equation that increases in height of the confined water layer lead to higher diffusion coefficients, while increasing its viscosity leads to lower values.

1.4 The effect of ionic strength on lipid bilayers

Many researchers have focused on the effects of ionic strength on the lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) due to its abundance in natural cell membranes. This zwitterionic lipid contains both a positive and negative charge that results in net charge neutrality. The effect of ionic strength on the structure of POPC lipid bilayers in multilamellar systems has been studied extensively both experimentally and through computer simulations, but conflicting results have been reported.

In 2003 fluorescence correlation spectroscopy (FCS) and molecular dynamics were used to study the effect of sodium chloride on fluorescently-labeled mulitlamellar POPC bilayers (Bockmann 2003). FCS results showed a very steep reduction in bilayer fluidity in the presence of small amounts of NaCl. The diffusion coefficient of the bilayers in 100 mM NaCl were found to be 50% lower than bilayers in 0 mM NaCl. However, virtually no changes in bilayer fluidity were observed as NaCl concentration was increased above 100 mM. Similar results were obtained when using both negatively charged head group labeled fluorescent dyes and zwitterionic acyl chain labeled dyes. Molecular dynamics (MD) simulations were performed that

produced results similar to those obtained through FCS (Bockmann 2003). The MD simulations suggested that sodium atoms are attracted to the electronegative carbonyl oxygens of the POPC lipids and serve as a linker, causing on average three POPC lipids to form a single large slow-moving complex. The authors argue that this model explains the sharp reduction in fluidity of the bilayers as NaCl concentration is increased. Similar results were achieved in MD simulations of POPC bilayers in the presence of the divalent cation Ca^{2+}, except it was found that on average the calcium ion would cause an average of four POPC lipids to bind together to form a larger complex (Bockmann 2004).

In 2008, molecular dynamics simulations examining the effect of NaCl and KCl on free-standing POPC bilayers were performed (Gurtovenko 2008). Similar to Bockmann et al (2003), it was found that small amounts of NaCl lead to steep reductions in the diffusion coefficients of bilayers and that the sodium atoms are strongly attracted to the carbonyl oxygens of POPC. Interestingly, KCl was found to have a much smaller (yet still significant) effect on bilayer fluidity than NaCl, and this was attributed to the fact that the potassium ion is larger than the sodium ion. It was found in both cases that the cations are responsible for the observed effects; the anions do not play a role. Similarly, MD simulations indicate the presence of cations enhances the ability of PC lipids to form lipid-lipid complexes in free-standing bilayers (Pandit 2003).

In 2009 pulsed field gradient NMR was used to study the diffusion of POPC lipids in a multilamellar system in the presence of NaCl and $CaCl_2$ (Filippov 2009). An advantage of this system is that no probe molecule is needed. In contrast to others (Bockmann 2003), no change in diffusion coefficient was observed when NaCl or $CaCl_2$ concentration was increased from 0 mM to 300 mM and 100 mM respectively.

The authors hypothesized that Bockmann's experimental results (Bockmann 2003) are an artifact of the fluorescent labels used.

Small-angle x-ray diffraction and other techniques to study the structure of POPC bilayers have found that monovalent and divalent salts only affect the structure of lipid bilayers at concentrations above one molar (Pabst 2007). At these high concentrations, the salts (especially $CaCl_2$), lead to more ordered rigid structures. Although diffusion measurements were not taken, this more rigid structure likely has less fluidity than bilayers in lower levels of salt.

In 2005, Monte Carlo simulations were used to observe the rigidification of a zwitterionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayer as monovalent salt concentration is increased (Kotulska 2005). Unlike POPC, this lipid is fully saturated so the chains can rotate and can pack more tightly together than unsaturated acyl chains. In contrast to the results of others (Bockmann 2003), in this work the observed rigidification was explained based purely on an ion condensation model. In this model, increased salt concentration leads to electrostatic screening of the charges on lipid head groups and allows the lipids to pack closer together. As the lipids pack closer together, van der Waals interactions between the acyl chains also increase.

In contrast to zwitterionic lipids such as POPC, there is much more agreement in the literature that even low levels of ionic strength have a strong impact on the structure of lipid bilayers containing anionic lipids. Anionic lipids are important physiologically, comprising between 1 – 17 % of the lipids in mammalian cell membranes (Hauser 1992). Even small amounts of monovalent salt have been found to markedly reduce the diffusion coefficient of anionic bilayers in multilamellar systems, with divalent salts producing an even stronger effect (Fillipov 2009). It has been shown in solid-supported lipid bilayers consisting of a mixture of zwitterionic

and anionic lipids that both monovalent and divalent salts can lead to lipid de-mixing creating areas that are enriched in one particular lipid (Cambrea 2007, Lamberson 2007). MD simulations of free-standing bilayers containing both zwitterionic and anionic lipids have also suggested sodium atoms binding the two types of lipids together into a larger complex (Pandit 2003).

Interestingly, it has been found that initial salt concentration at the time of bilayer formation can affect the symmetry/asymmetry of the anionic lipids in a bilayer. At a NaCl concentration of 75 mM, anionic lipids will repel the negatively charged glass substrate and be predominantly located in the outer leaflet of an SLB (Shreve 2008). While, at a NaCl concentration of 150 mM, sufficient electrostatic screening enables the symmetric distribution of anionic lipids during formation of the bilayer (Richter 2005). There are no published reports suggesting altering salt concentration after SLB formation would result in an alteration of anionic lipid distribution. Presumably, the energy required for lipid flip-flop between leaflets is too high. If however, there is lipid asymmetry present in a bilayer, reports have shown that the fluidity of the individual leaflets of a SLB are virtually identical (Zhang 2005). Additionally, spectroscopic studies have found altering the bulk salt concentration above a solid-supported lipid bilayer has little effect on the structure of the confined water layer between the bottom leaflet and the support (Kim 2001).

1.5 Polyelectrolyte layers

The negative charge present on glass surfaces at neutral pH prevents the formation of highly anionic solid-supported lipid bilayers directly on glass due to charge repulsion. However, applying a positively charged polyelectrolyte layer to glass will enable the formation of highly anionic lipid bilayers. Experiments conducted with highly anionic membranes could make it easier for researchers to isolate and examine the role of anionic lipids in physiological membranes.

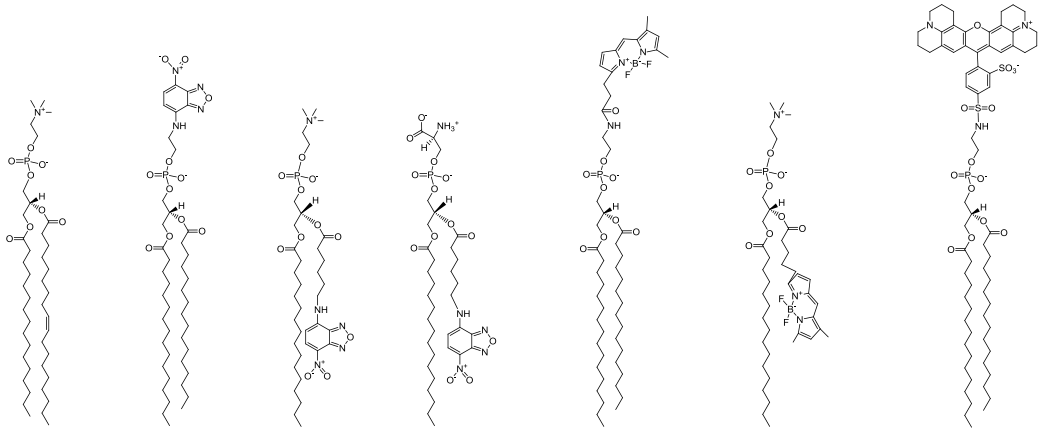
An additional advantage of using polyelectrolyte layers is that alternating positive and negative polyelectrolyte layers can be applied to glass in a reliable and predictable way (Decher 1997). This property allows one to control the thickness of polyelectrolyte layers and therefore, the space between the bilayer and glass substrate. While the 1 nm space typically between the bilayer and substrate of solid supported lipid bilayers (devoid of polyelectrolyte) provides adequate space for lipids to move freely, it is not conducive to the mobility of larger species such as transmembrane proteins (Koenig 1996, Bayerl 1990, Johnson 1991, Tanaka 2005). Forming bilayers on polyelectrolyte layers can enhance the mobility of Preliminary results presented here demonstrate that ionic strength affects the fluidity of bilayers formed on polyelectrolyte cushions. larger species by reducing the frictional coupling of the species with the support (Tanaka 2005, Sackmann 1996).

CHAPTER 2.

MATERIALS AND METHODS

2.1 Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0-18:1 POPC),1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (16:0-18:1 POPS), and 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphoserine (16:0-06:0 NBD PS) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Fluorescently labeled lipids, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (BODIPY FL DHPE), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (β-BODIPY FL C_5-HPC), Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (Texas Red DHPE), N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (NBD-PE), and 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD C_6-HPC) were purchased from Invitrogen (Carlsbad, California). Buffer salts (sodium chloride, calcium chloride, and Tris) were purchased from BDH and Bio-Rad. Whatman nuclepore track-etch membranes with a pore size of 100 nm were used in vesicle extrusion. Glass coverslips used as solid supports for the bilayers were purchased from VWR. Sulfuric acid and hydrogen peroxide used in glass surface preparation were purchased from BDH and Sigma-Aldrich. Polydiallyldimethylammonium (PDDA) was purchased from Polysciences (Warrington, Pennsylvania). Polydimethylsiloxane (PDMS; Sylgard 184) was obtained from Dow Corning.



POPC **NBD-head** **NBD-tail** **NBD-tail PS** **BODIPY-head** **BODIPY-tail** **Texas Red-head**

Figure 3: Chemical structures of the lipids used in this work and the short-hand designations used in the text for them listed underneath the structure. From left to right: POPC (zwitterionic), NBD PC (zwitterionic), NBD C_6-HPC (zwitterionic), NBD PS (anionic), BODIPY FL DHPE (anionic), BODIPY FL-C_5HPC (zwitterionic), and Texas Red DHPE (anionic).

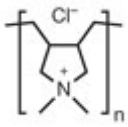


Figure 4: Structure of Polydiallyldimethylammonium (PDDA).

2.2 Vesicle preparation

All lipids and additives were used as received without further purification. Lipids and additives were dissolved in chloroform at appropriate ratios to obtain the desired composition in the vesicles. 0.1 mol% fluorescently labeled lipids were added as a fluorescent marker for the diffusion measurements in all bilayers. Chloroform solutions were dried under a stream of nitrogen gas for approximately five to ten minutes to remove the bulk of the chloroform. The lipids were then placed under vacuum for approximately three hours to remove any remaining chloroform. The POPS-containing formulations were resuspended in a Tris-buffered solution (6.42 mM Tris, 0 mM NaCl, pH 7.2) and POPC vesicles were resuspended in a Tris-buffered saline solution (6.42 mM Tris, 150 mM NaCl, pH 7.2). All buffers were prepared

with purified water with a resistance of at least 18.2 MΩ*cm. The aqueous lipid solutions were subjected to five freeze-thaw cycles by alternating between rapid freezing in liquid nitrogen and thawing in a lukewarm (40 ^{o}C) water bath. Finally, vesicle solutions were passed five times through a 100 nm polycarbonate filter using an extruder (Northern Lipids, CA).

2.3 Support preparation

Freshly cleaned glass coverslips were prepared daily. Coverslips were placed in a ceramic boat and soaked in piranha solution made of 70%/30% (v/v) concentrated H_2SO_4 and 50% H_2O_2 for ten minutes. The coverslips were then serially diluted with purified water to remove the piranha solution and then stored in purified water until use. Immediately before use, the coverslips were dried with ultra high purity nitrogen gas.

To form a positively-charged polyelectrolyte layer on glass (which was used as a support for both zwitterionic and negatively-charged bilayers for some experiments), 200 µl of a 0.1mg/ml solution of PDDA (MW 40,000) in water was deposited onto a glass coverslip inside a PDMS well. After a ten minute incubation period, the well was rinsed for one minute with a Tris-buffered solution (6.42 mM Tris, pH 7.2) containing 150 mM NaCl (for POPC bilayer formation) or 500mM NaCl (for POPS-containing bilayer formation).

2.4 Supported-lipid bilayer preparation

To form a 99.9 mole percent POPC bilayer directly on glass, 200 µl of vesicles in a Tris-buffered saline solution (6.42 mM Tris, 150 mM NaCl, pH 7.2) at a concentration of 0.25 mg/ml were deposited inside a PDMS well adhered to a glass coverslip. After a ten minute incubation period, the bilayer was rinsed with a Tris-buffered saline solution (6.42 mM Tris, 150 mM NaCl, pH 7.2) for one minute to remove excess vesicles from the well. The bilayer was then gently scratched with a

dissection tool to remove small portions of the bilayer to aid in focusing the bilayer on the microscope. Following the scratch step, an additional one minute rinse using the previous buffer was performed. To form a POPC (zwitterionic) bilayer on a PDDA layer an identical procedure to that described above was used, except instead of ten minute vesicle incubation, the vesicles were incubated for one hour.

To form POPS containing bilayers on PDDA, 200 μl of 75%/25% POPS/POPC vesicles in a Tris-buffered saline-free solution (6.42 mM Tris, 0 mM NaCl, pH 7.2) at a concentration of 0.33 mg/ml was added to the PDMS well containing the PDDA layer. After a 60 minute incubation period, the bilayer was rinsed for one minute with the Tris-buffered solution containing the desired saline concentration. The bilayer was then scratched with a dissection tool to aid in focusing. An additional one minute rinse using a Tris-buffered saline solution at the desired NaCl concentration followed the scratch step.

Following the bilayer preparation, diffusion measurements were taken. All reported diffusion coefficients result from averaging at least three measurements on each sample. After measurements were taken, the saline buffer inside the sample was exchanged by rinsing with the desired saline buffer for one minute, then new diffusion measurements were taken.

2.5 Diffusion measurements using fluorescence recovery after photobleaching (FRAP)

An inverted Zeiss Axiovert Observer. Z1 microscope equipped with a Hammamatsu EM-CCD camera (model C9100-13) and a CVI Melles Griot argon-krypton tunable laser (model 643-AP-A01) were used to carry out FRAP experiments. Experiments were conducted at 10x magnification.

A “Smart Experiments” program in Zeiss Axiovision software was used to automate bleaching of the bilayer and image acquisition. First, an image of the

unbleached bilayer was taken. Next, the bilayer was bleached with a circular laser beam for 200 ms using an excitation wavelength of 488 nm for green dyes or 568 nm for red dyes, creating a bleach spot approximately 10 μm in diameter. Post bleach, images were acquired every two seconds for one minute then every ten seconds for eight minutes. By comparing the fluorescence intensity of the area of the bleach spot with the fluorescence intensity of a non-bleached area of the bilayer over time it was possible to account for photobleaching of the sample. Matlab software was used to fit the recovery curves and calculate diffusion coefficients using the following equations (Axelrod 1976, Gordon 1995):

$$F(t) = \theta F_i f(t) + (1-\theta)F(0) \tag{3}$$

$$f(t) = F_i \nu K^{-\nu} \Gamma(\nu) P(2K|2\nu) \tag{4}$$

$$\nu = \left(1 + \frac{2t}{\tau_D}\right)^{-1} \tag{5}$$

$$F(0) = F_i[1 - e^{-K}]/K \tag{6}$$

$$\tau_D = \frac{w^2}{4D} \tag{7}$$

where *F(t)*is the fluorescence signal as a function of time, θ is the mobile fraction, F_i is the fluorescence of the bleach spot before it is bleached, F(0) is the fluorescence of the bleach spot immediately after bleach, *K* is a parameter that describes bleach depth, Γ is the gamma function, P is the χ^2 probability function, τ_D is the time constant, w is the radius of the bleach spot (as defined by Axelrod), and *D* is the diffusion coefficient.

2.6 Ion binding to PS calculations

The following reaction for ion binding was assumed:

$$ion + lipid \leftrightarrow Complex \tag{8}$$

With an association constant given by:

$$K_a = \frac{[Complex]}{[ion][lipid]} \quad (9)$$

This equation was then rearranged to find:

$$\%\ unbound\ lipid = 1 - \frac{[ion]K_a}{1+[ion]K_a} \quad (10)$$

Values for the association constant were taken from the literature (Ohki 1981, Pandit 2003, and Feigenson 1986).

2.7 Atomic force microscopy (AFM) measurements

An Agilent Technologies PicoPlus AFM (Santa Clara, CA) was used to measure roughness of glass and PDDA layers submerged under water. Magnetic dynamic (AC) mode was used. Rectangular silicon nitride cantilevers from Molecular Imaging (Tempe, Arizona) with a force constant of 0.02 N/m and a resonant frequency of 15 kHz were used. First order background subtraction was applied to all images to account for sample tilt.

2.8 Vesicle sizing

A Zetasizer Nano (Malvern Instruments, Worcestershire, UK) was used to determine particle size using dynamic light scattering. Plastibrand disposable cuvettes (model 7591 70) were used and samples were equilibrated at 25^{o}C for two minutes before measurements were taken. Five measurements were taken and averaged for each data point presented.

CHAPTER 3.

RESULTS AND DISCUSSION

3.1 Effect of NaCl on fluorescent labels in POPC bilayers formed on glass

Solid-supported lipid bilayers consisting of 99.9 mole percent POPC and 0.1 mole percent fluorescently labeled lipid were successfully formed on glass microscope coverslips. Negatively charged head group labeled lipids (BODIPY FL DHPE, Texas red DHPE, and NBD-PE), zwitterionic acyl chain labeled lipids (BODIPY FL C_5-HPC and NBD C_6-HPC), and a negatively charged acyl chain labeled lipid (NBD PS) were used. The structures of these lipids are presented in Chapter 2: Materials and Methods. Fluorescence recovery after photobleaching (FRAP) was used to obtain three diffusion measurements at different locations in a sample at a specific NaCl concentration. The buffer was then exchanged with one containing a different NaCl concentration, and the process was repeated for several NaCl concentrations (Figure 5).

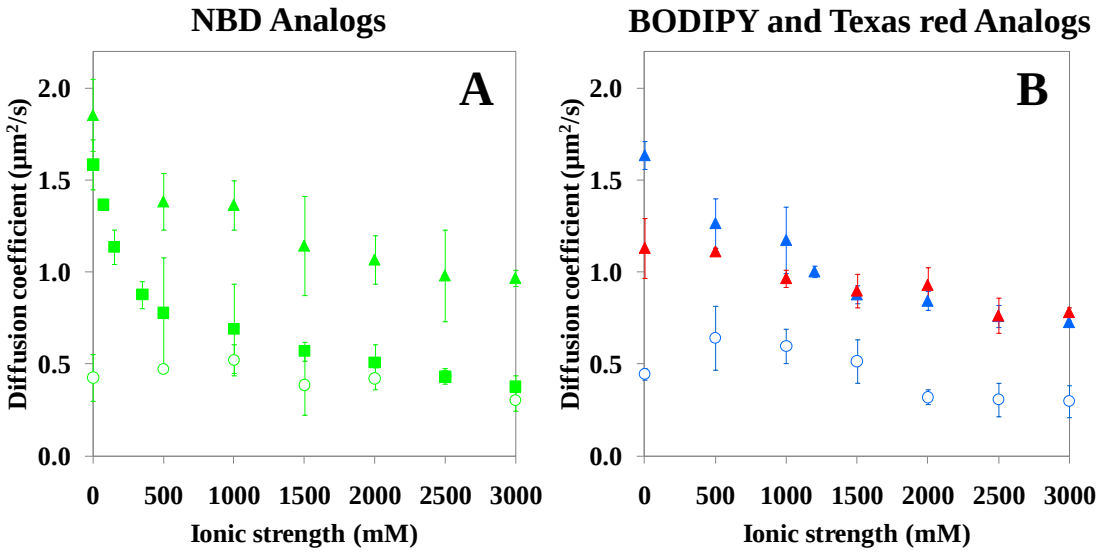


Figure 5: Comparison of fluorescently-labeled lipid diffusion in a background of zwitterionic 99.9% POPC bilayers formed on glass as a function of NaCl ionic strength in Tris-buffered aqueous solutions. Fluorescent lipids comprised 0.1 mol% of the bilayer. (A) NBD-head (*green triangles,* ▲), NBD-tail PS (*green squares,* ■) and NBD-tail (*green circles,* ○). (B) BODIPY-head (*blue triangles,* ▲), BODIPY-tail (*green circles,* ○), and Texas Red-head (*red triangles,* ▲).

It was found that when using negatively charged dyes located in the lipid head group region, the measured diffusion coefficient which is relatively high at low NaCl concentration decreases significantly as NaCl concentration is increased. However, increasing NaCl concentration from 0 to 3000 mM was found to have minimal impact on the lower diffusion coefficients of the samples containing fluorescent labels in the acyl chain region of zwitterionic lipids. These trends were not dependent on the specific identity of the dye. In order to determine if this large distinction in behavior of the two types of lipids is due to differences in dye location or charge, the effect of NaCl concentration on NBD-tail PS was examined. This acyl chain labeled anionic lipid was found to have a high diffusion coefficient at 0 mM NaCl that is reduced as NaCl concentration is increased, similarly to the anionic head labeled lipids. This result indicates that the charge of the lipid examined (and not the dye location) is

responsible for the relatively high NaCl sensitive diffusion coefficients of the head labeled lipids.

The relative constancy of the diffusion measurements of bilayers containing acyl chain labeled dyes makes it likely that NaCl concentrations of up to 3000 mM have only a small impact on the fluidity of solid-supported POPC bilayers. Otherwise, a drop in the fluidity of the zwitterionic labels would have been observed. This is in agreement with what others have reported for zwitterionic diffusion coefficients in multilamellar systems (Filippov 2009, Pabst 2007). Although there are seemingly contradictory published reports on the impact of an acyl chain labeled dye on the diffusion of a lipid, some authors hypothesize that a dye label in the viscous membrane environment will result in low levels of lipid fluidity (Bockmann 2003). This is in agreement with equation 2. This hypothesis implies that the fluidity of the zwitterionic acyl chain labeled lipids is limited by the presence of the acyl chain labels, and could explain the high sensitivity to NaCl observed in the case of the NBD tail PS lipid observed at low salt that diminished at higher salt. At low NaCl concentrations, electrostatic repulsion minimizes the interaction of the acyl chain label with surrounding lipids, but as salt concentration is increased the combined effects of effects electrostatic screening in the head group regions and the viscous drag of the acyl chain label begins to emerge.

The data indicates that the negatively charged head group labeled dyes are not accurately reporting the fluidity of the POPC background. While zwitterionic lipids are unaffected by NaCl, NaCl does have a significant impact on the mobility of negatively charged lipids. The negative charge present could cause the head-labeled lipids to repel one another, leading to higher levels of fluidity than is observed in the case of zwitterionic lipids. As salt is added, electrostatic shielding reduces the repulsive forces present while enhancing acyl chain van der Waals interactions and the

lipids are able to pack closer together creating a more rigid system (Filippov 2009, Pabst 2007). In this ion condensation model, one would expect the fluidity of the anionic lipids to be similar to the fluidity of the zwitterionic lipids at high NaCl concentrations as is observed. As an alternative to the ion condensation model, in the ion binding model the sodium ion is acting as a linker allowing anionic labeled lipids to bind with other labeled lipids or with POPC molecules. This would create larger slower moving complexes (Bockmann 2003, Gurtovenko 2008, Mukhopadhyay 2004, Pandit 2003). Although the distinction is subtle, in the presented data there appears to be a negative correlation between head group size and the impact of NaCl concentration, with lipids labeled with large head groups demonstrating fluidity that is less sensitive to NaCl concentration. This is in agreement with the hypothesis of an ion binding multiple lipids together since steric hindrance would likely make it more difficult for lipids with large head groups to bind with one another. Ion binding is thought to occur at the carbonyl oxygen atom of lipids, and therefore the acyl chains of the participating lipids need to be in very close proximity in order for ion binding to occur. If the lipids involved have large head groups, it could become more difficult for the lipids involved to attain close spatial proximity.

To rule out the possibility that NaCl sensitive electrostatic repulsion between the anionic lipids and the negatively charged glass substrate leads to increased bilayer-substrate spacing and higher diffusion coefficients (as equation 2 predicts), the following experiment was conducted. An anionic head group labeled dye and zwitterionic acyl chain labeled dye were both incorporated into a single POPC bilayer, and the diffusion coefficient of each was measured (Figure 6).

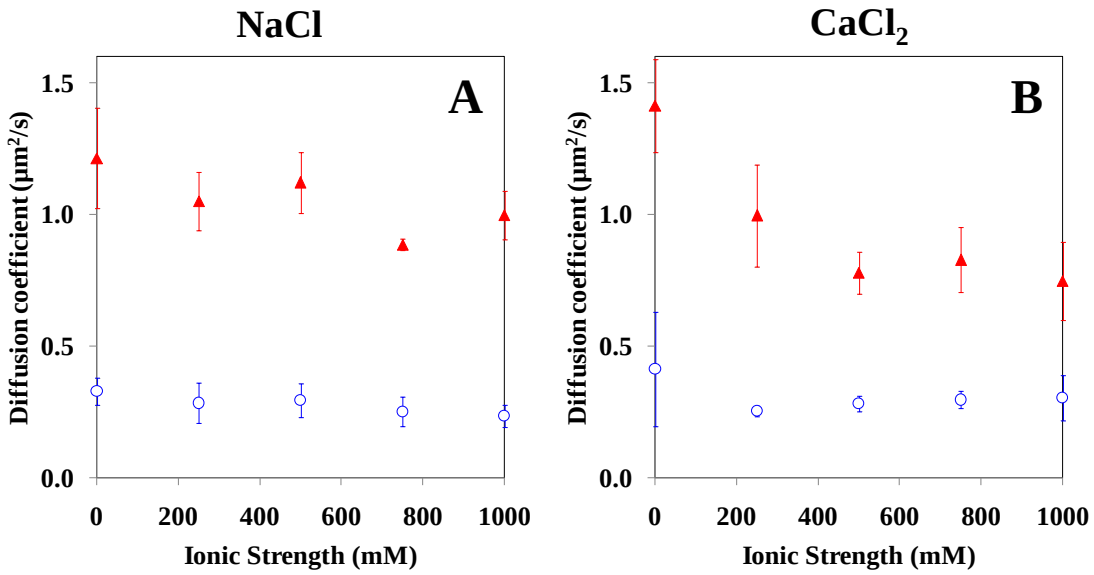


Figure 6: Diffusion coefficients measured by FRAP of a zwitterionic 99.9% POPC bilayer sample formed on glass as a function of (A) NaCl and (B) $CaCl_2$ ionic strength in Tris-buffered aqueous solutions containing a mixture of 0.1 mol% Texas red DHPE (*red triangles*) and 0.1 mol% BODIPY-tail (*blue circles*) .

It was found that a head group labeled dye and an acyl chain labeled dye in the same bilayer report different diffusion coefficients compared to each other, but similar to their individual values obtained when only one dye is present. This result suggests that the bilayer-substrate spacing of POPC bilayers labeled with anionic lipids is similar to that of POPC bilayers labeled with a zwitterionic lipid. Additionally, as was discussed previously, altering bulk salt concentration has been found to have little impact on the structure of the confined water layer and its viscosity (Kim 2001). Light scattering experiments of vesicles discussed later independently support that NaCl changes the area per lipid and supports the ion binding and/or condensation mechanisms.

3.2 Effect of NaCl incubation time on head group labeled POPC bilayers formed on glass

To assess the impact of NaCl incubation time on the measured diffusion coefficient of a POPC bilayer containing a head group labeled dye, measurements of the diffusion coefficient of a bilayer at a specific NaCl concentration and spatial location were taken repeatedly over the course of 64 minutes (Figure 7). These experiments were conducted because under certain conditions it has been found that changing the salt concentration of the buffer surrounding a solid-supported lipid bilayer can create temporary three-dimensional structural protrusions (Cambrea 2007). The presence of these structural features could affect the measured diffusion coefficient of the lipid bilayers.

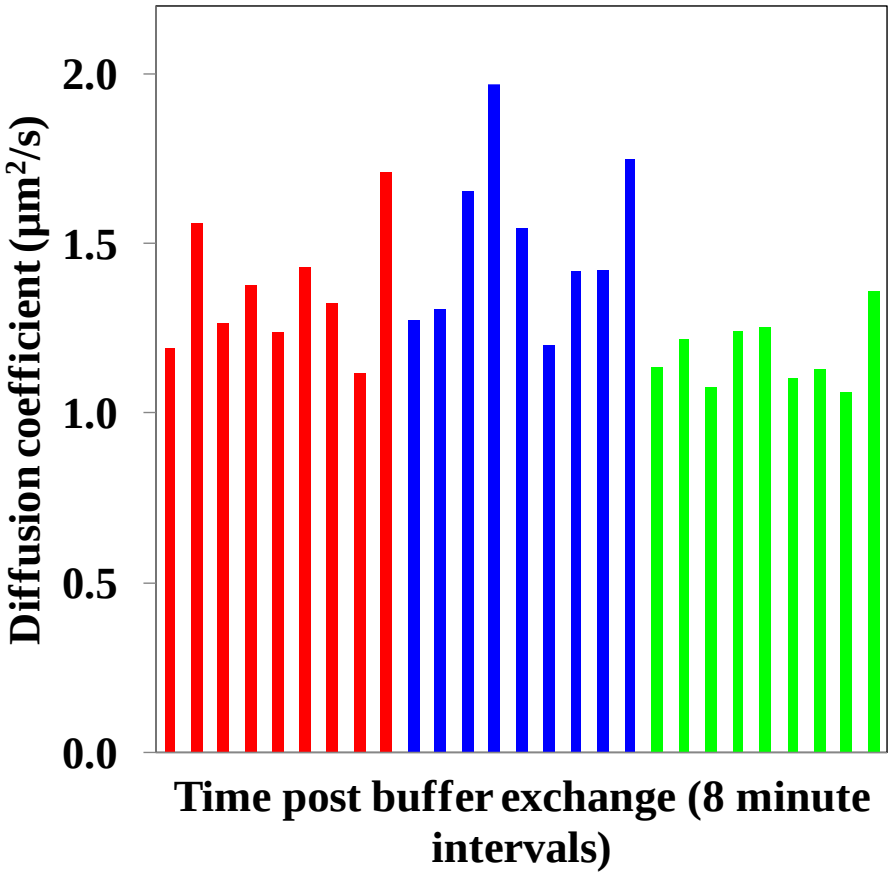


Figure 7: Effect of NaCl incubation time on the measured diffusion coefficient of a 99.9 mole percent POPC and 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer. Measurements were taken every eight minutes for 64 minutes at each NaCl concentration: 500mM NaCl (red),150mM NaCl (blue), and 1000mM NaCl (green).

There does not appear to be any trends in the collected data. Therefore, it appears that ion exchange occurs within the eight minutes and longer incubation times have little or no impact. The variability in the data can be attributed to the intrinsic variability of the measurement technique. To assess this variability, the diffusion coefficients at each salt concentration were averaged and the standard deviation was calculated (Figure 8). The precision of the measurement technique was found to be very high at approximately ± 0.2 $\mu m^2/s$.

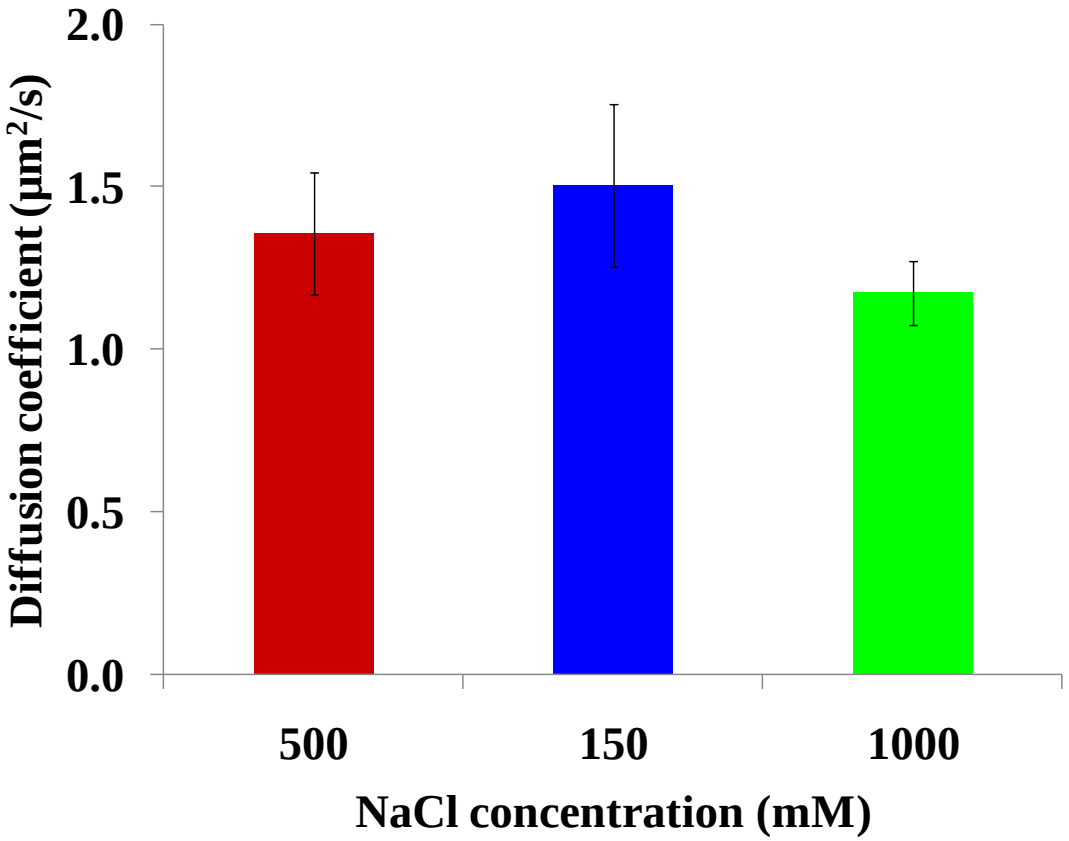


Figure 8: Effect of NaCl concentration on the measured diffusion coefficient of a 99.9 mole percent POPC 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer formed on glass. Nine measurements at the same spatial location of the bilayers were performed for each NaCl concentration tested.

3.3 Effect of $CaCl_2$ on POPC bilayers formed on glass

To further investigate the impact of salt on the observed diffusion coefficient of a POPC bilayer labeled with an anionic lipid, the effect of a divalent salt was assessed (Figure 9). To control for the debye length in comparing the effect of NaCl and $CaCl_2$, ionic strength was calculated using the following equation:

$$Ionic\ strength\ (mM) = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2 \qquad (11)$$

where c_i is the molar concentration and z_i is the charge of the ith ion

Based on this equation, the molarity of an NaCl solution is equal to its ionic strength. A NaCl solution however, must have a molar concentration three times higher than a $CaCl_2$ solution in order to have the same level of ionic strength.

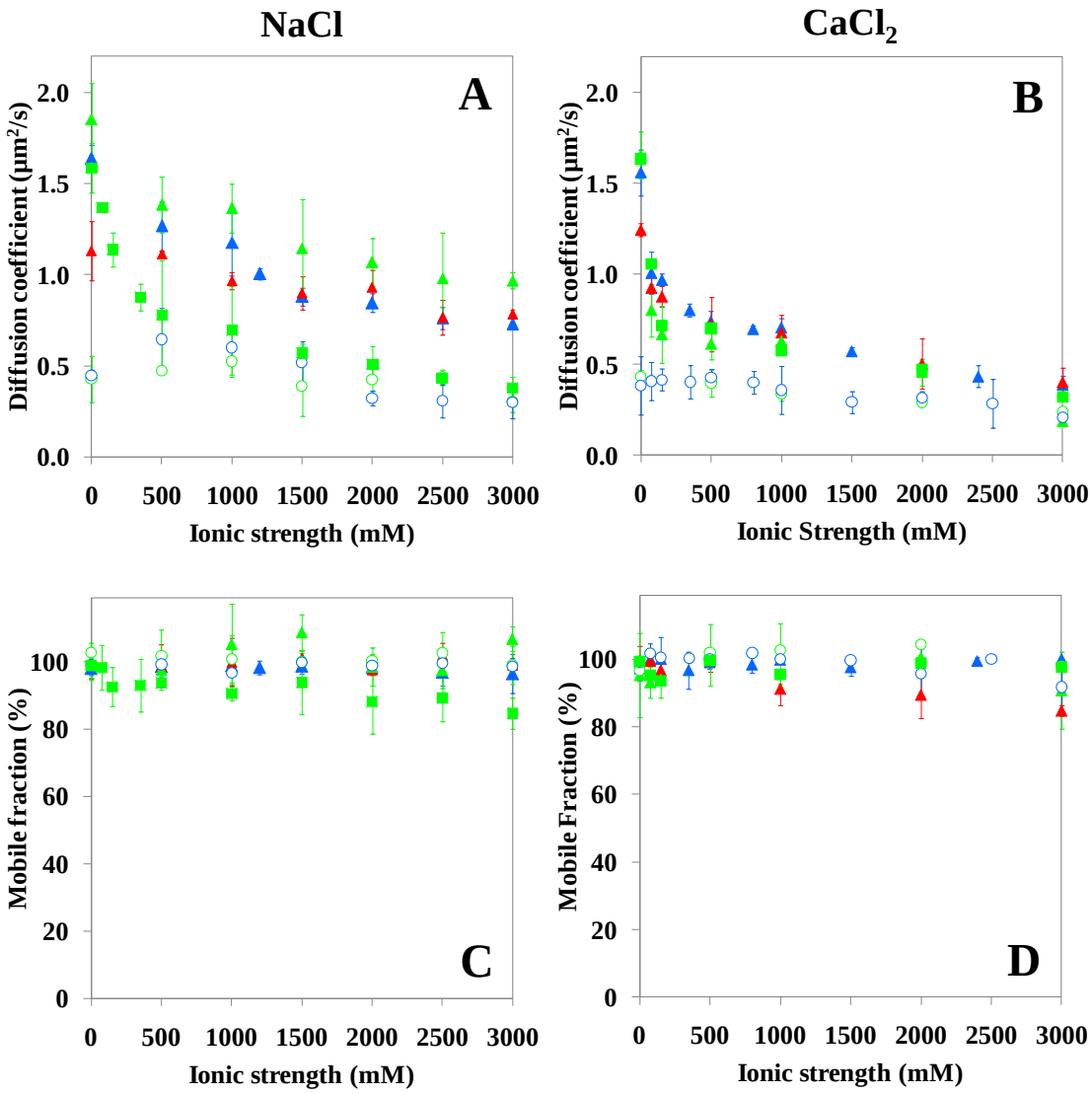


Figure 9: Comparison of lipid diffusion in zwitterionic 99.9% POPC bilayers formed on glass as a function of (A) NaCl and (B) $CaCl_2$ ionic strength in Tris-buffered aqueous solutions with 0.1% labeled lipid. NBD-head (*green triangles,* ▲), NBD-tail PS (*green squares,* ■), NBD-tail (*green circles,* ○), BODIPY-head (*blue triangles,* ▲), BODIPY-tail (*blue circles,* ○), and Texas Red-head (*red triangles,* ▲).

It was found that for a given ionic strength, the divalent salt consistently resulted in a lower diffusion coefficient than the monovalent salt for anionic lipids. Unlike sodium, as calcium concentration is increased, the fluidity levels of all of the anionic lipids are suddenly reduced to values similar to the zwitterionic tail-labeled lipids. This corroborates that the divalent salt is interacting much more strongly with the anionic lipids than the monovalent salt (Bockmann 2004, Filippov 2009, Pabst 2007)). It has been shown that in some cases, divalent salts cause negatively charged lipids to de-mix from zwitterionic ones in a solid supported lipid bilayer, while monovalent salts will not (Lamberson, 2007). Therefore, the calcium based ion binding of multiple anionic lipids could explain the large drop in measured bilayer fluidity as $CaCl_2$ concentration is increased. As with the monovalent salt, the divalent salt appears to have minimal effects on the fluidity levels of zwitterionic lipids and all samples were found to have high mobile fractions.

3.4 Reversibility of the effect of ionic strength on head group labeled POPC bilayers formed on glass

To determine if the effect of salt on a head group labeled dye is reversible, a single sample was twice cycled through three different NaCl concentrations and the diffusion coefficient was measured after each buffer exchange (Figure 10 A). In the case of NaCl it can be seen that initially at a concentration of 500 mM the sample was found to have a diffusion coefficient of approximately 1.4 $\mu m^2/s$. After the buffer was exchanged to 150 mM and then 0 mM, the diffusion coefficient progressively increased. The buffer was then exchanged to 150 mM, 500 mM, and 0 mM. At each of these three NaCl concentrations, the diffusion coefficient at a particular NaCl concentration was found to be very similar to the value initially measured. This indicates that the effect of NaCl on the sample is reversible. It also indicates that the interaction of the sodium chloride with the bilayer is relatively weak since this salt can

be readily removed. Interestingly, very similar results were obtained in the case of the divalent salt, $CaCl_2$ (Figure 10 B). Diffusion coefficient measurements at a particular divalent salt concentration were found to be reproducible despite multiple buffer exchanges. Although the divalent salt is interacting more strongly with the bilayer than the monovalent salt at a given ionic strength (as shown by the lower diffusion coefficients obtained with the divalent salt), it is still easily removed from the bilayer.

One sample was cycled through both NaCl and $CaCl_2$ (Figure 10 C). Diffusion coefficients were found to be similar to those obtained when only one type of salt was utilized. It was again found that the effect of the salts on the bilayer are reversible. The initial diffusion measurement at 0 mM salt was very similar to the final measurement at 0 mM salt after five NaCl/$CaCl_2$ buffer exchanges took place. It was found that the NaCl is easily exchanged with $CaCl_2$ and that $CaCl_2$ is easily removed with a buffer at 0 mM salt.

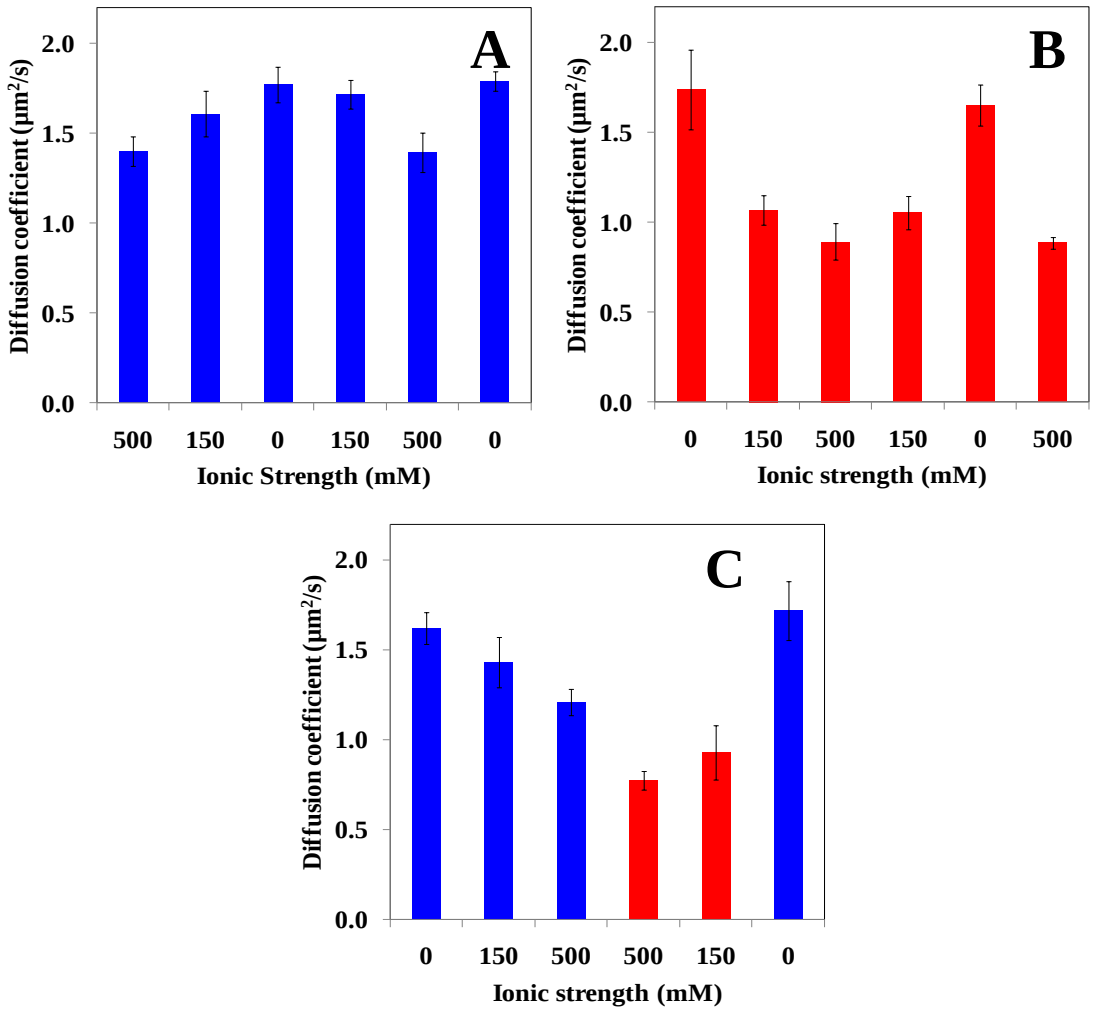


Figure 10: The diffusion coefficient of lipids in a 99.9% POPC bilayer labeled with 0.1 mol% BODIPY-head bilayer formed on glass as a function of ionic strength of NaCl or $CaCl_2$ salts in Tris-buffered aqueous solutions. These experiments were conducted in the order they are presented in each graph, from left to right. (A) NaCl, (B) $CaCl_2$, (C) NaCl (*blue bars*) and $CaCl_2$ (*red bars*).

Images of a bilayer cycled through the same salt conditions as Figure 10 C are presented at magnifications of 10x (Figure 11) and 100x (Figure 12). At 10x magnification, the cycling of buffer salts causes no visible damage. At 100x magnification, as $CaCl_2$ solutions are exchanged for the bulk solution we occasionally observe small defects that occur predominantly near the scratched areas of the bilayer. These defects however, were no longer visible after the $CaCl_2$ solution was exchanged for a salt free buffer. It is likely that the strong interactions the $CaCl_2$ has with the

anionic lipids causes the lipids to pack closer together promoting defect formation. Removing the $CaCl_2$ reduces the packing density of the lipids and leads to removal of the defects. To confirm that defect formation was actually occurring (as opposed to domain formation), vesicles added to defect containing bilayers were also found to repair the defects.

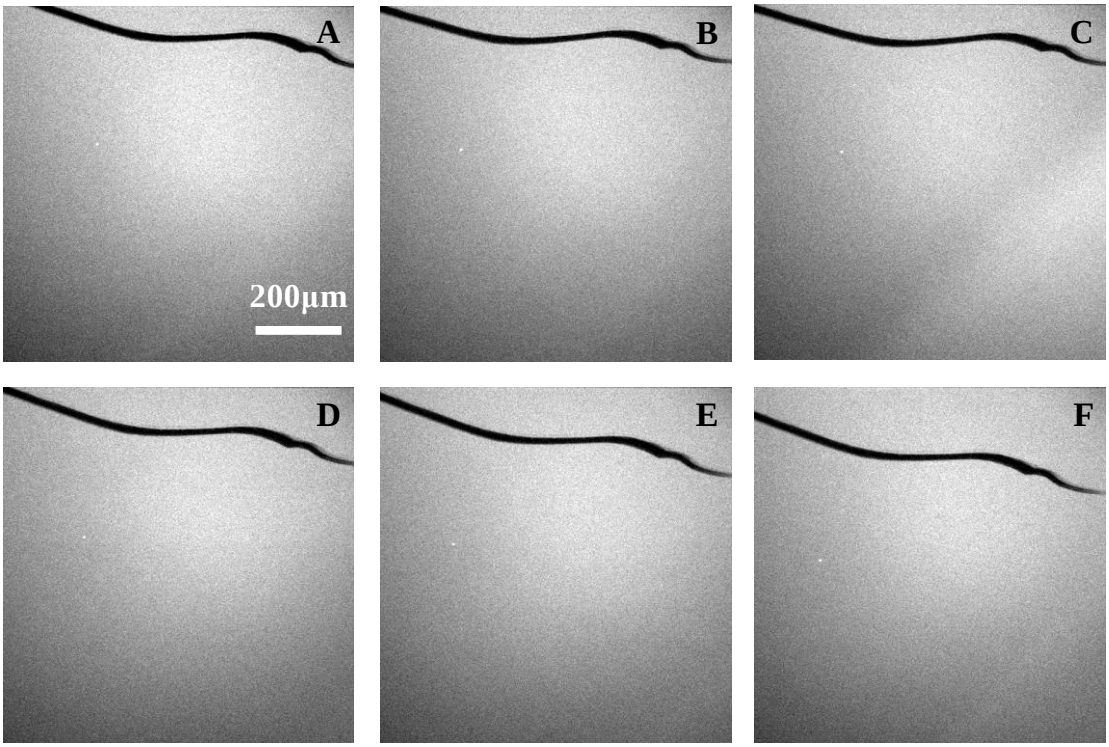


Figure 11: Images of a 99.9% POPC bilayer labeled with 0.1 mol% BODIPY-head that is cycled through NaCl and $CaCl_2$ solutions that correspond to those used in Fig. 4 *C*. These images at 10x magnification show that there is no visible damage to the bilayer. The black line is a scratch made with a dissection tool to aid in focusing on the bilayer. (A) 0 mM, (B) 150 mM NaCl, (C) 500 mM NaCl, (D) 167 mM $CaCl_2$, (E) 50 mM $CaCl_2$, (F) 0 mM.

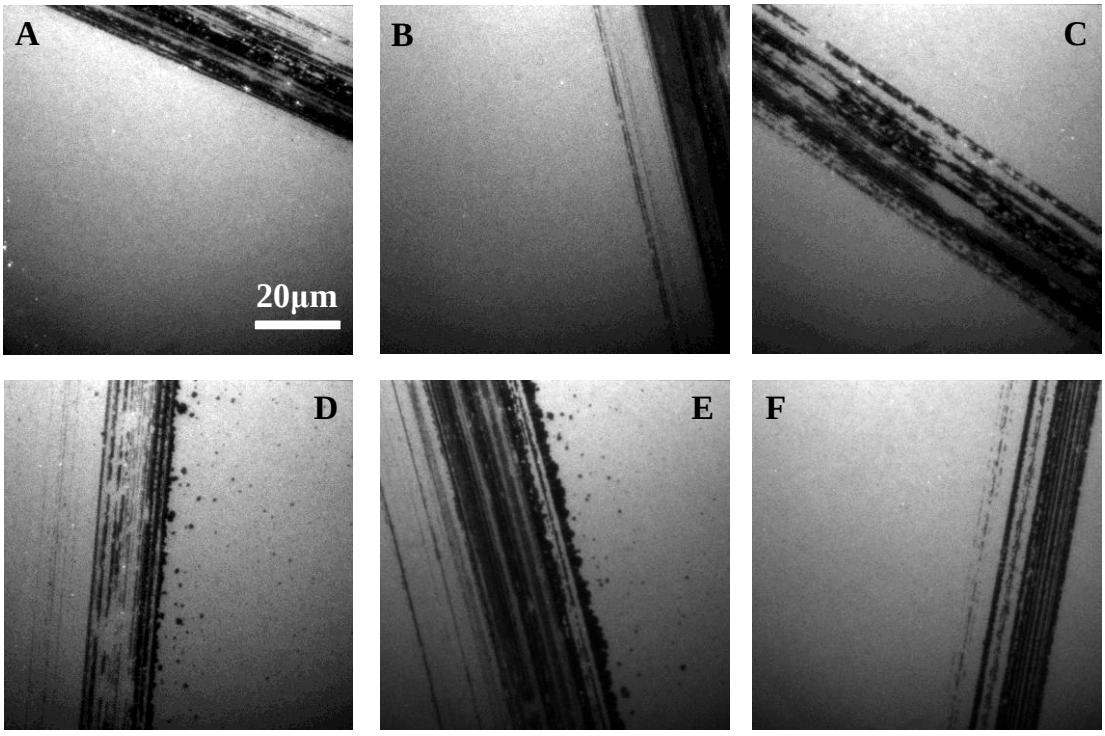


FIGURE 12: Images of a 99.9% POPC bilayer labeled with 0.1 mol% BODIPY-head that is cycled through NaCl and $CaCl_2$ solutions that correspond to those used in Fig. 4 *C*. These images at 100x magnification show small defects that occur as the bilayer is cycled through $CaCl_2$ solutions. The black line is a scratch made with a dissection tool to aid in focusing on the bilayer. (A) 0 mM, (B) 150 mM NaCl, (C) 500 mM NaCl, (D) 167 mM $CaCl_2$, (E) 50 mM $CaCl_2$, (F) 0 mM.

3.5 Sodium and Calcium ion binding calculations for PS

Since sodium and calcium binding constants with PS have been published in the literature (Ohki 1981, Pandit 2003, Feigenson 1986), it is possible to calculate the percentage of unbound anionic lipid present at a given salt concentration (Figure 13 and Figure 14). Since varying association constants have been reported in the literature, calculations for multiple association constants were performed. In these figures the empty shapes show the calculated percentage of unbound anionic lipid (left y-axis) for a given ionic strength and association constant. The solid black circles show the experimentally normalized diffusion coefficient (right y-axis) of the NBD-tail PS lipid as a function of ionic strength. For both the monovalent and divalent salt there was found to be a strong nearly one to one correlation between the amount of bound ions and the reduction in NBD tail PS diffusion coefficient. This correlation supports the ion binding hypothesis.

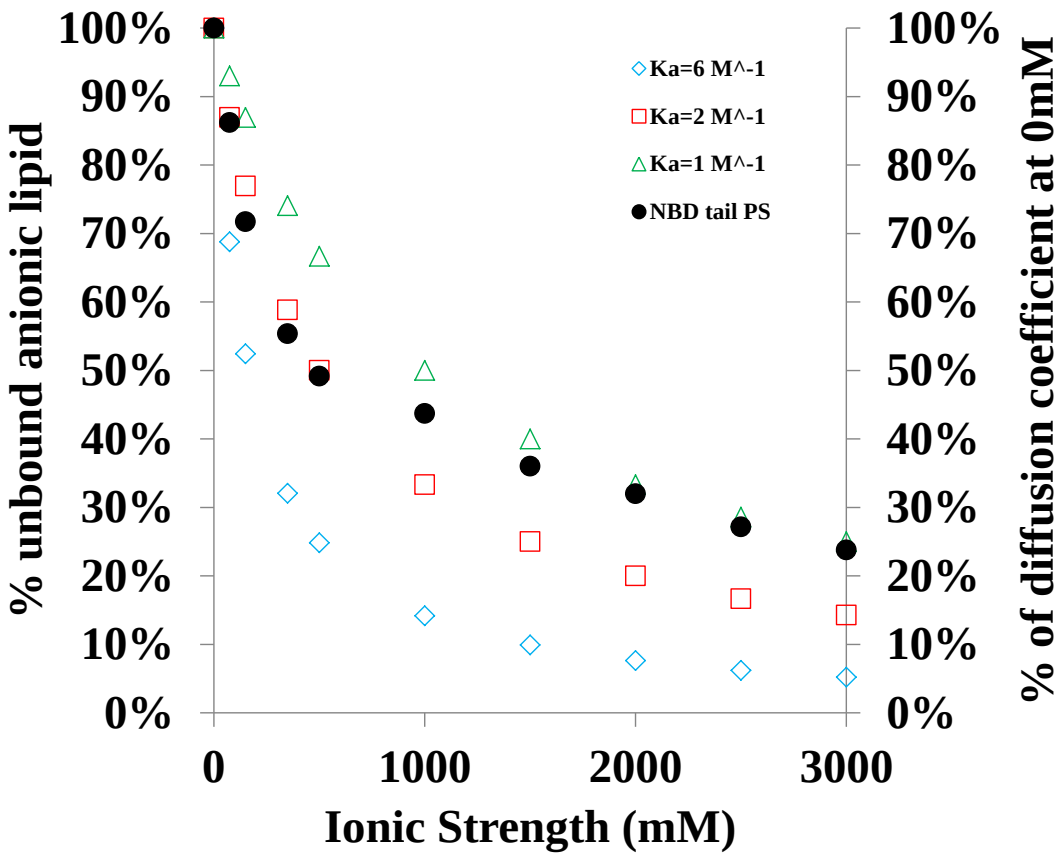


Figure 13: Calculated sodium binding to PS plotted using the left y-axis using three different association constants from the literature: blue diamonds (Pandit 2003), red squares (Ohki 1981), and green triangles (Feigenson 1986).The normalized diffusion coefficient of NBD tail PS (solid black circles) is plotted using the right y-axis.

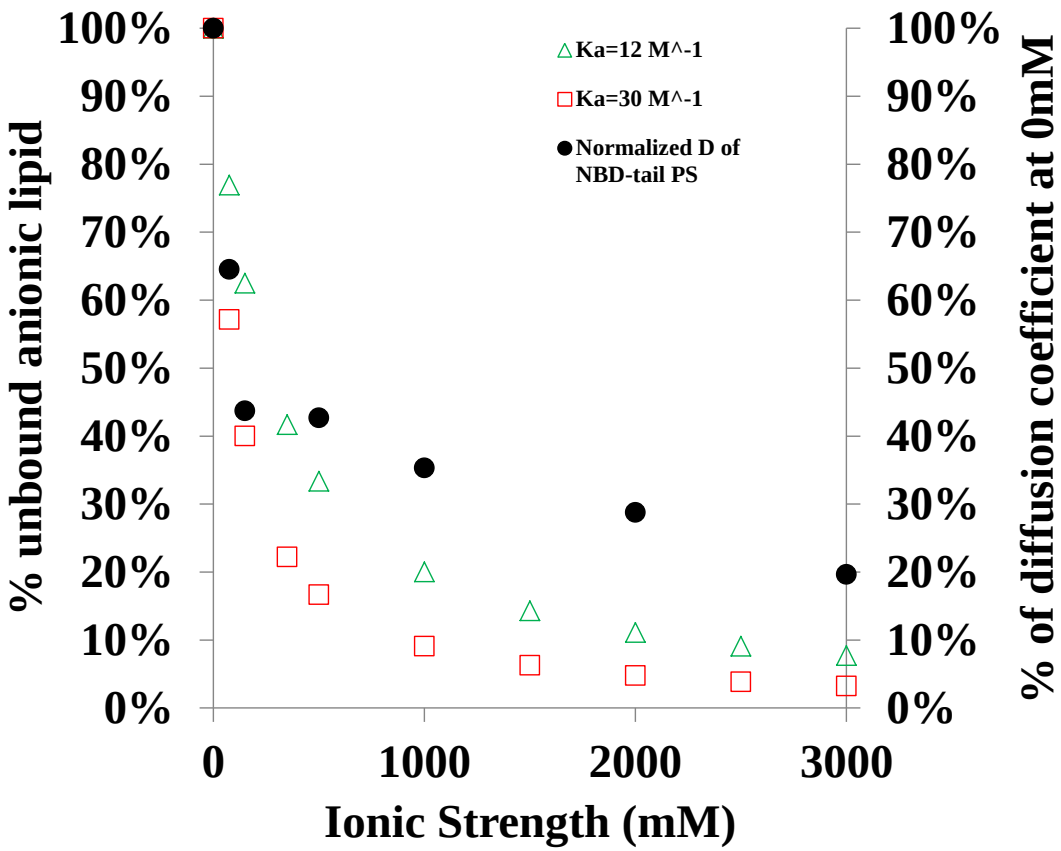


Figure 14: Calculated calcium binding to PS plotted using the left y-axis using two different association constants from the literature: green triangles (Ohki 1981) and red squares (Feigenson 1986).The normalized diffusion coefficient of NBD tail PS (solid black circles) is plotted using the right y-axis.

3.6 Effect of NaCl on head group labeled POPS vesicles

Experimental evidence presented here and by others, along with computer simulations, indicate that NaCl has stronger interactions with negatively charged lipids than zwitterionic ones (Filippov 2009, Mukhopadhyay 2004, Eisenberg 1979). Light scattering measurements were taken in order to observe this effect. First, 99.9% POPS 0.1% BODIPY-head vesicles with an internal NaCl and glucose concentration of 0 mM were examined (Figure 15). It was found that increasing the external NaCl concentration of the vesicles from 0 mM to 135 mM resulted in an immediate reduction in hydrodynamic diameter of approximately twenty percent. To determine if this reduction was due to the osmotic gradient created by the NaCl, a sample was examined with a comparable external concentration of glucose in place of NaCl. This sample however, was found to yield very similar measurements to the sample with an external NaCl/glucose concentration of 0 mM. Additionally, all samples were measured 24 hours after the external buffer had been exchanged, and these measurements were found to be very similar to measurements taken immediately following external buffer exchange (t = 0 hrs). The data collected suggests that the reduction in hydrodynamic diameter of POPS vesicles observed in the presence of an external NaCl concentration results from the interaction of the ions present with the vesicles. This interaction occurs quickly and does not change over the course of 24 hours. One possibility is that the NaCl is allowing the POPS lipids to pack closer together.

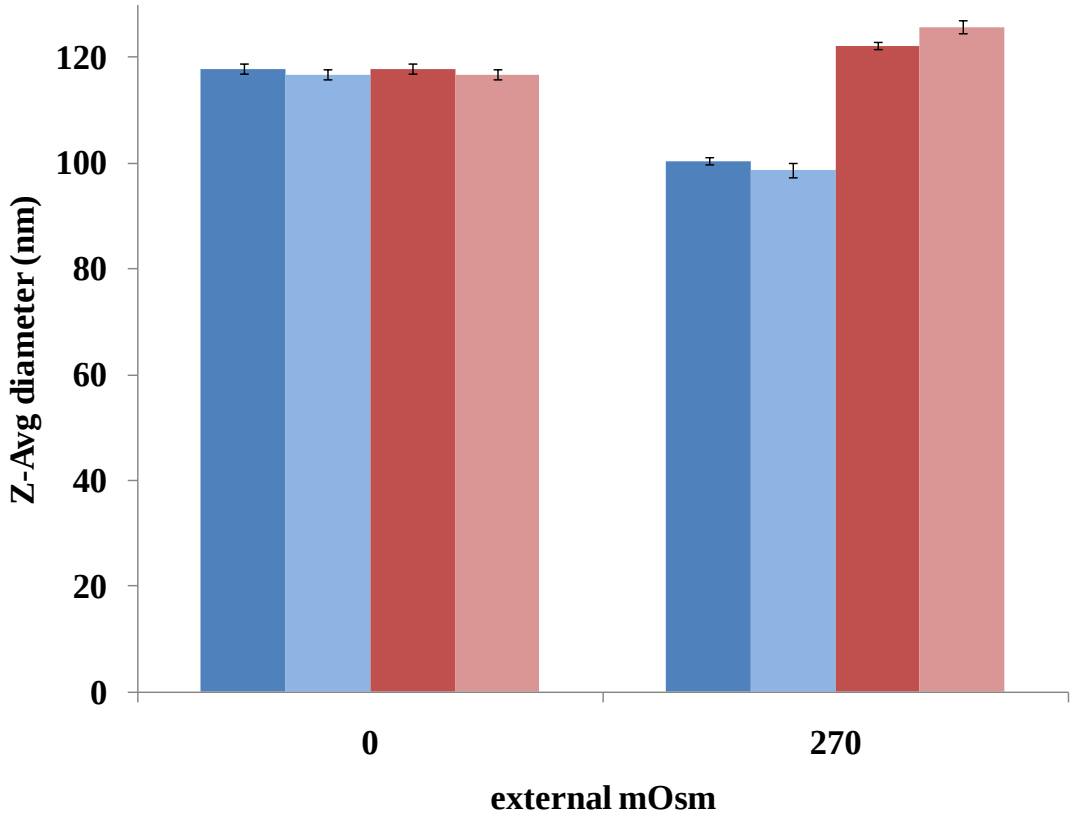


Figure 15: Light scattering measurements showing the effect of external NaCl and glucose concentration on 99.9 mole percent POPS 0.1 mole percent Bodipy FL DHPE vesicles with an internal NaCl/glucose concentration of 0mM (similar results are achieved when using 75 mole percent POPS 25 mole percent POPC 0.1 mole percent Bodipy FL DHPE vesicles). NaCl at t = 0hrs (dark blue ■), NaCl at t = 24hrs (light blue ■), glucose at t = 0hrs (dark red ■), and glucose at t = 24hrs (light red ■).

In order to examine the reversibility of the effect of NaCl on POPS vesicles, a single sample of vesicles was cycled through multiple external NaCl concentrations (Figure 16). It was found that increasing external NaCl concentration of the POPS vesicles from 0 mM to 135 mM once again resulted in a reduction of hydrodynamic size of approximately 20%. Reducing the external NaCl concentration of this sample however, lead to an increase in hydrodynamic size. Nearly full recovery of the initial size was achieved.

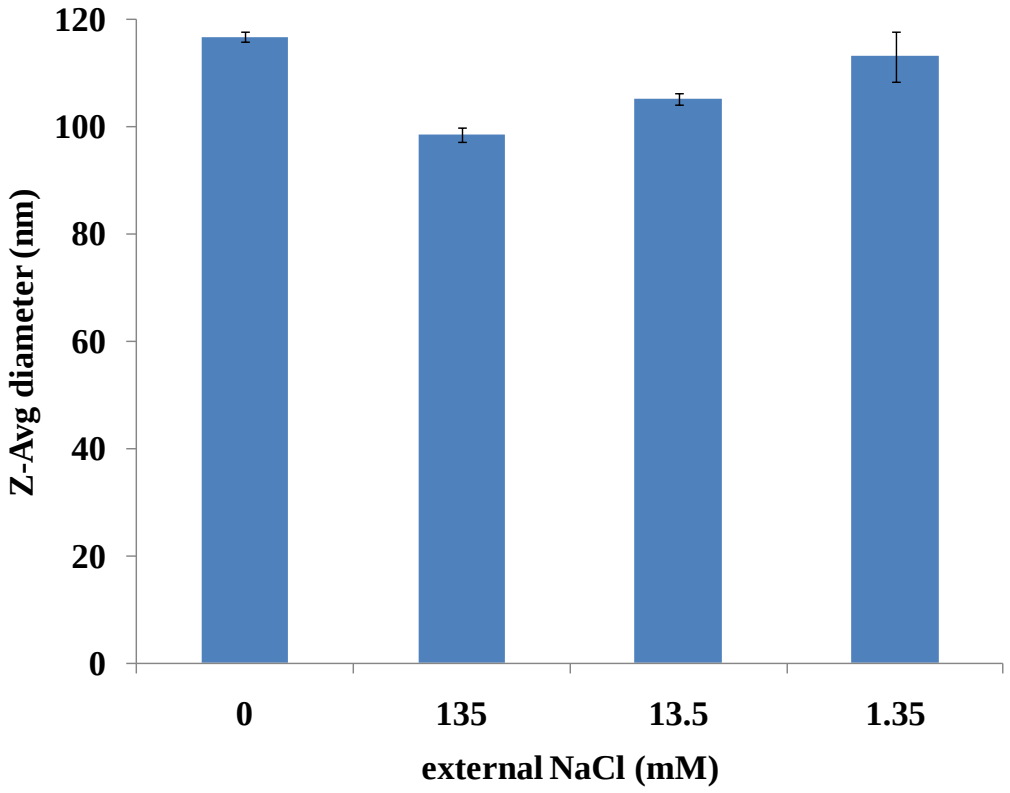


Figure 16: Light scattering measurements showing the reversible effect of external NaCl on 99.9 mole percent POPS 0.1 mole percent Bodipy FL DHPE vesicles with an internal NaCl concentration of 0 mM. One aliquot of vesicles was cycled through 0,135,13.5, and 1.35 mM NaCl by serial dilution.

POPC vesicles were also examined in the presence of an external NaCl concentration of 135 mM NaCl (Figure 17). Unlike POPS vesicles, the external NaCl concentration had no detectable effect on the hydrodynamic size of the POPC vesicles.

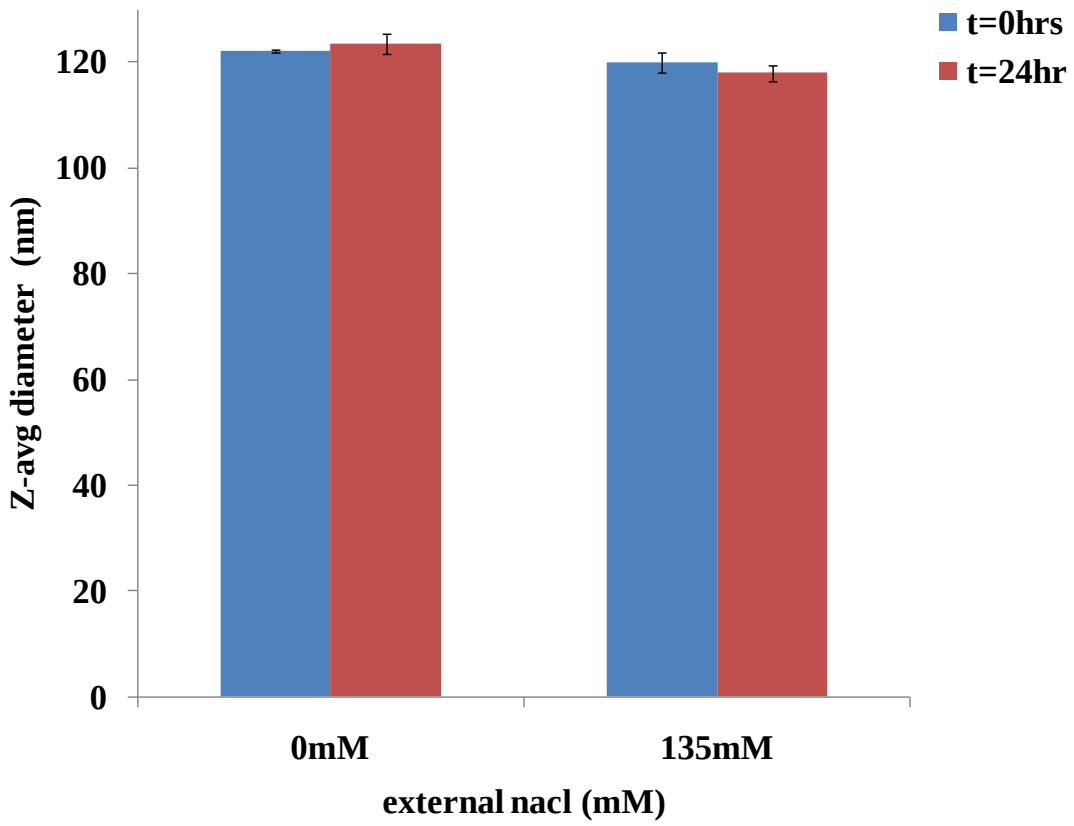


Figure 17: Light scattering measurements showing the effect of external NaCl concentration on 100 mole percent POPC vesicles with an internal NaCl concentration of 0mM.

The light scattering measurements presented here are in agreement with the FRAP measurements of solid-supported lipid bilayers. NaCl was found to have a significant, quick, and reversible impact on anionic lipids, but it was found to have no impact on zwitterionic lipids.

3.7 Preliminary experiments: Effect of NaCl on head group labeled POPC bilayers formed on PDDA

POPC bilayers with a negatively charged head group label were successfully formed on positively charged PDDA polyelectrolyte cushions. The diffusion coefficient of one of these bilayers was measured at different NaCl concentrations (Figure 18).

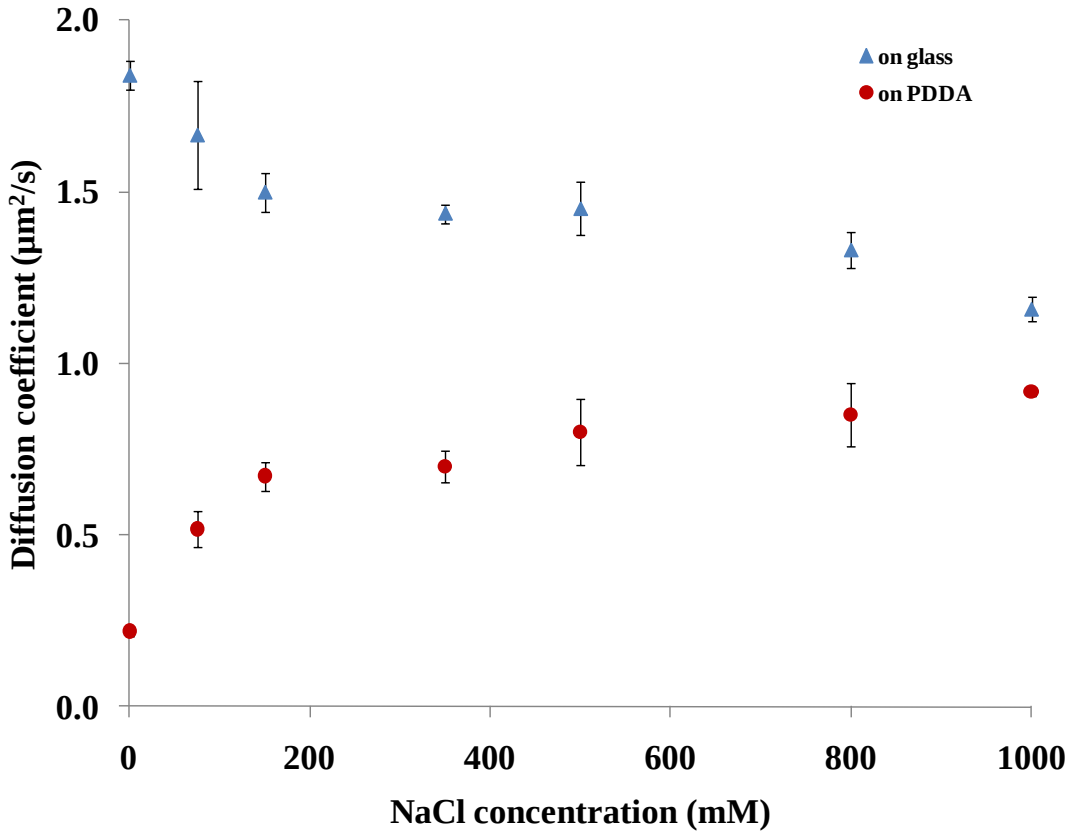


Figure 18: Effect of NaCl concentration on the measured diffusion coefficient of a 99.9 mole percent POPC and 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer on glass and on a PDDA layer.

Although it was found that higher NaCl concentrations lead to lower measured diffusion coefficients for POPC head group labeled bilayers on glass, the opposite trend was observed for identical bilayers formed on PDDA. This could be due to electrostatic attraction of the negatively charged head group label to the positively

charged polyelectrolyte. At low salt concentration the attraction is strong and reduces the mobility of the head group labels, while at high salt concentration increased electrostatic shielding reduces the attractive force (Zhang, 2000). Alternately, it is possible the polyelectrolyte layer is expanding as NaCl concentration is increased which leads to reduced frictional coupling of the bilayer with the solid support (Dubas 2001). To examine the effect of NaCl on the roughness of the PDDA, atomic force microscopy (AFM) measurements of PDDA roughness in various salt concentrations were taken (Figure 19).

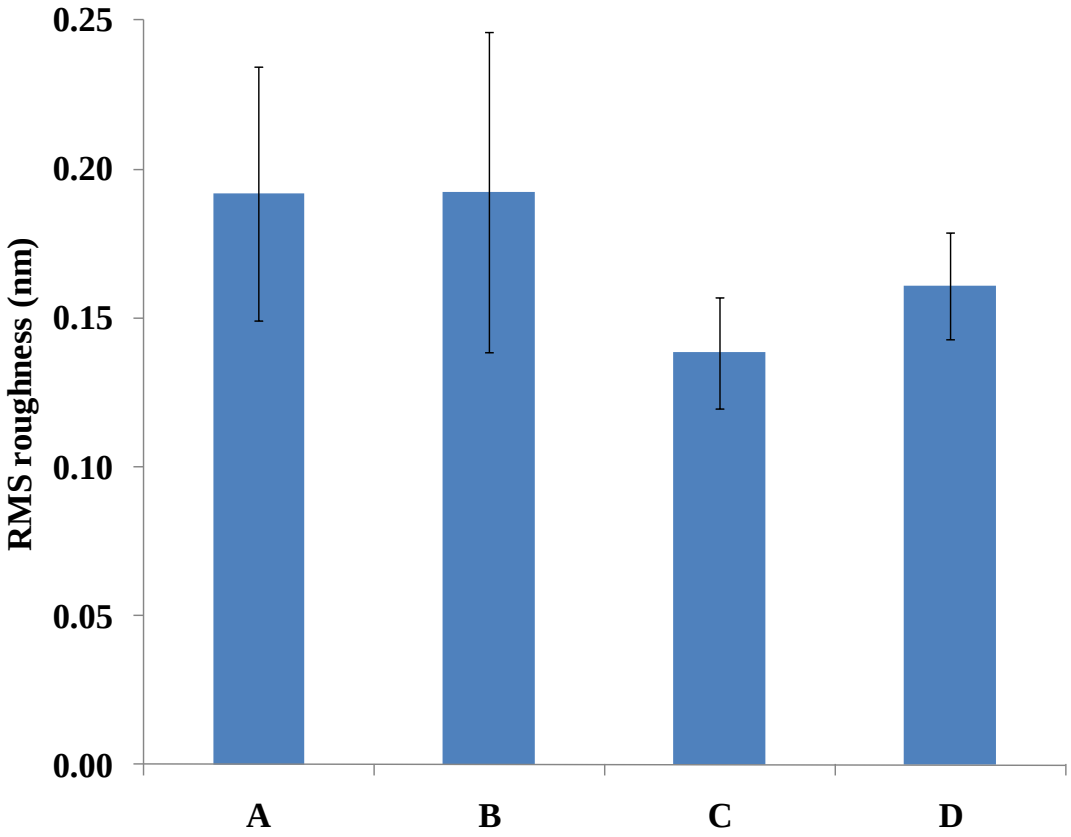


Figure 19: AFM roughness measurements of PDDA as a function of NaCl. (A) Glass only in water , (B) PDDA on glass in water, (C) PDDA on glass in 150mM NaCl buffer, (D) PDDA on glass in 500mM NaCl buffer.

AFM measurements were not able to detect a significant effect of NaCl on PDDA roughness. Therefore, it is unlikely that salt induced changes in polyelectrolyte

roughness are responsible for the change in bilayer fluidity observed as bulk salt concentration is altered.

3.8 Preliminary experiments: Effect of NaCl on head group labeled POPS-POPC bilayers formed on PDDA

POPS is an anionic lipid commonly found in mammalian cells. Because of its negative charge however, bilayers containing high concentrations of POPS cannot be directly formed on glass. These bilayers can be formed on a positively charged PDDA layer.

Bilayers containing POPS were successfully formed on PDDA polyelectrolyte layers. It was found that the POPS containing bilayers behaved similarly to the POPC bilayers in that higher salt concentrations lead to higher measured diffusion coefficients (Figure 20). As with the POPC bilayers, it is possible that the NaCl is altering the attraction of the negative head group labeled lipid to the PDDA layer, and this is causing the increased observed fluidity as NaCl concentration is increased. It was found however, that the POPS containing bilayer yielded higher diffusion coefficients than the POPC bilayer at all the NaCl concentrations tested. The presence of a large amount of negatively charged lipid in the POPS sample could be reducing the likelihood that the negatively charged dye molecules are being attracted to the PDDA layer. This would lead to higher reported diffusion coefficients.

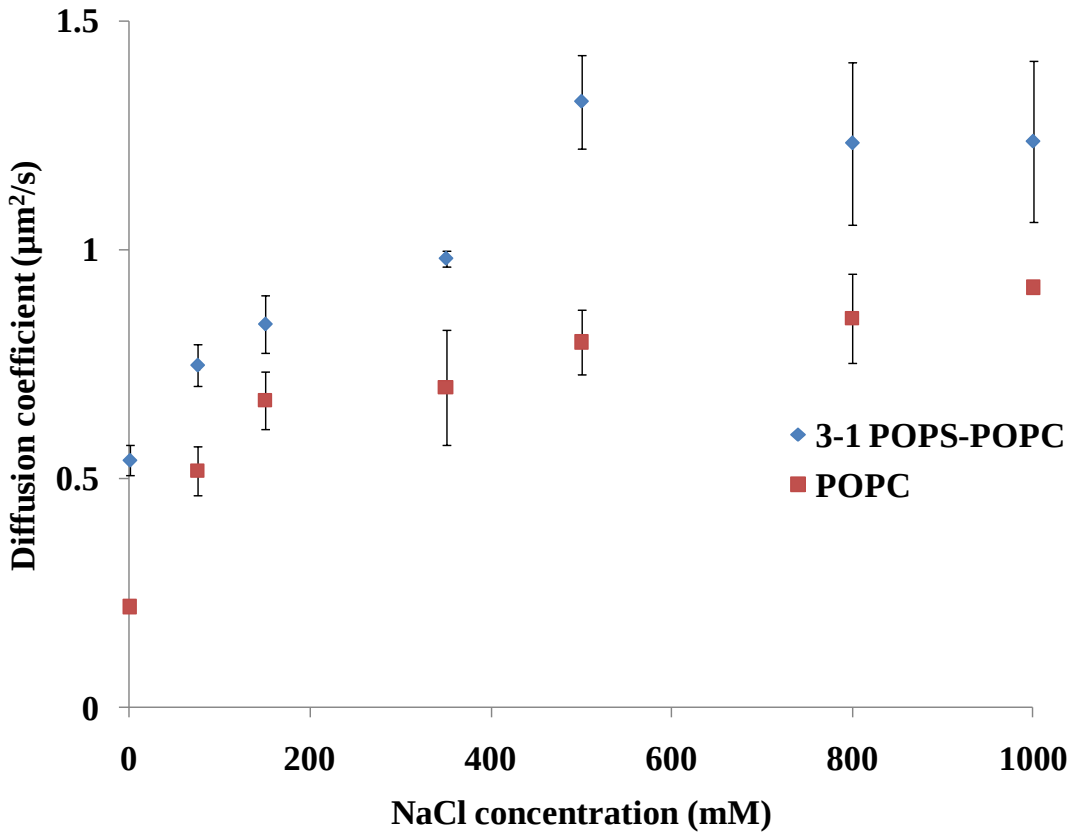


Figure 20: Effect of NaCl concentration on the measured diffusion coefficient of a 75 mole percent POPS 25 mole percent POPC 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer and 99.9 mole percent POPC 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer on a PDDA layer.

A POPS bilayer formed on PDDA was twice cycled through three different NaCl concentrations (Figure 21). As with the POPC bilayers formed on glass, it was found that the NaCl effect on the measured diffusion coefficients of POPS bilayers formed on PDDA were also reversible. Diffusion coefficients at a particular NaCl concentration were found to be unaffected by cycling the sample through multiple NaCl concentrations.

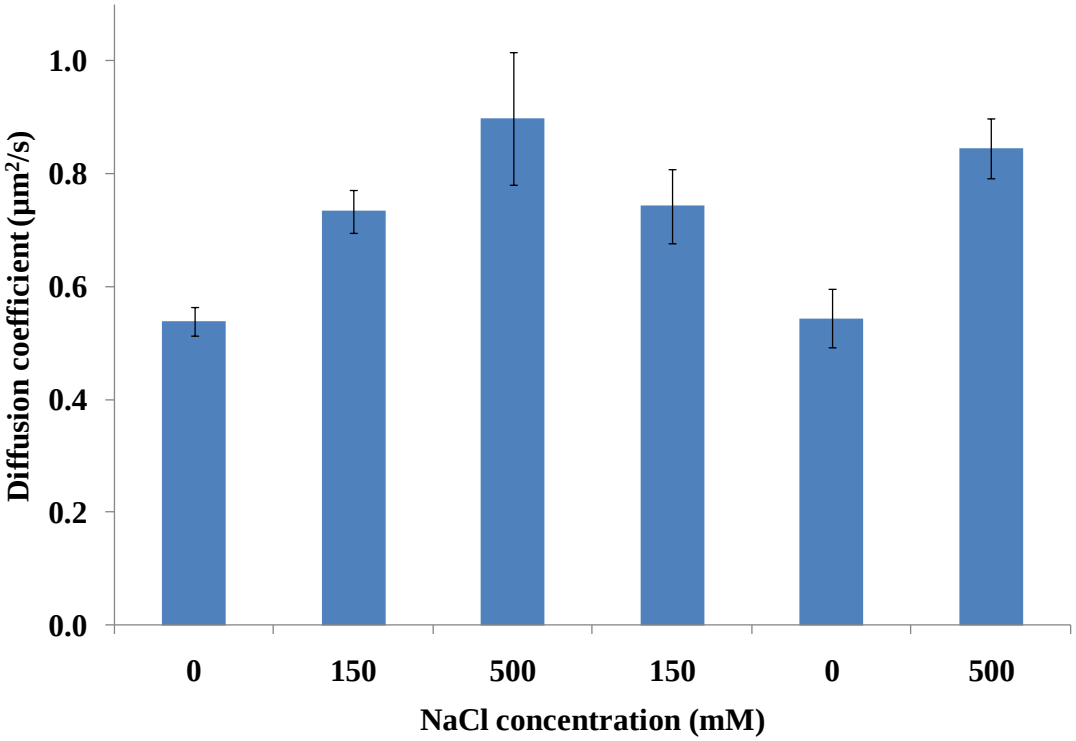


Figure 21: Effect of NaCl concentration on the measured diffusion coefficient of a 75 mole percent POPS 25 mole percent POPC and 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer.

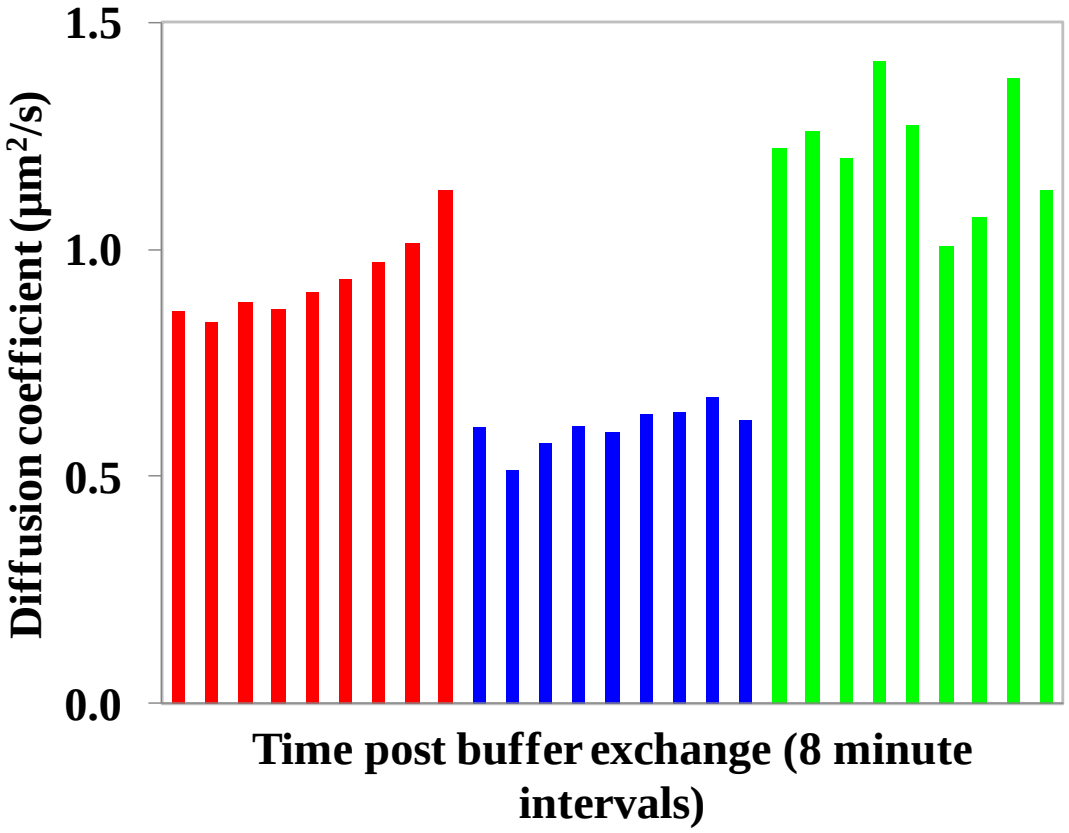


Figure 22: Effect of NaCl incubation time on the measured diffusion coefficient of a 75 mole percent POPS 25 mole percent POPC and 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer. Measurements were taken every eight minutes for 64 minutes at each NaCl concentration: 500mM NaCl (red),150mM NaCl (blue), and 1000mM NaCl (green).

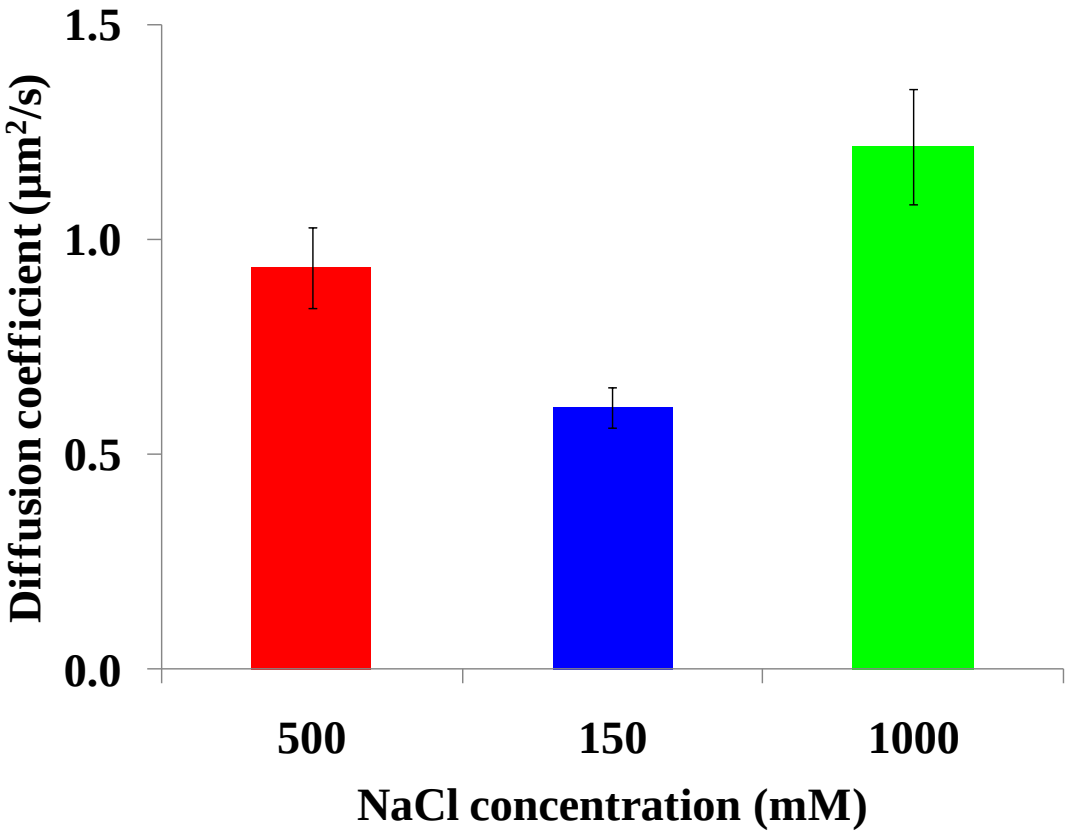


Figure 23: Effect of NaCl concentration on the measured diffusion coefficient of a 75 mole percent POPS 25 mole percent POPC and 0.1 mole percent Bodipy FL DHPE labeled lipid bilayer formed on glass. Nine measurements at the same spatial location of the bilayers were performed for each NaCl concentration tested.

To assess the impact of NaCl incubation time on the measured diffusion coefficient of a POPS bilayer containing a head group labeled dye on a PDDA layer, measurements of the diffusion coefficient of a bilayer at a specific NaCl concentration and spatial location were taken repeatedly over the course of 64 minutes (Figure 22). A consistent trend of NaCl incubation time and measured diffusion coefficient was not detected. Therefore, NaCl incubation time was not found to impact the measured diffusion coefficient of a POPS bilayer on PDDA. The diffusion coefficients at each salt concentration were averaged and the standard deviation was calculated (Figure 23). The precision of the measurement technique was once again found to be very high.

CHAPTER 4.

CONCLUSIONS AND FUTURE DIRECTIONS

In this work it has been shown that ionic strength has very little impact on the fluidity of solid-supported lipid bilayers composed entirely of zwitterionic lipids. In a bilayer containing both zwitterionic and anionic lipids however, even low levels of ionic strength significantly reduced the fluidity of the anionic lipids, with divalent salt having a much stronger impact than a monovalent one. This occurred despite the very low amount (0.1%) of anionic lipid present. These results were supported by light scattering measurements of vesicles that indicate ions have strong interactions with anionic lipids, but not zwitterionic ones. This is in agreement with previously reported results using less physiologically relevant multilamellar bilayer systems (Filippov 2009).

The results presented here have many implications, among them: 1.) It is possible that the affect of ionic strength on anionic lipid fluidity observed in the solid-supported lipid bilayer system is also occurring in natural cell membranes and could be enhancing certain processes such as protein or lipid aggregation. 2.) Those studying the fluidity of membranes (whether natural or artificial) using techniques such as FRAP or FCS should be aware of the effect of ionic strength on the fluorescent probe being used. Measured diffusion coefficients could be an artifact of the probe molecule. 3.) Ionic strength can be used to control the fluidity of lipid bilayers. This could be an advantage in experiments where interactions between membrane components are being studied, since ionic strength could be used to increase or decrease bilayer fluidity and alter the rate of component interactions.

Preliminary experiments examining the effect of ionic strength on bilayers formed on polyelectrolyte layers were also conducted. Interestingly, mixed zwitterionic/anionic lipid containing membranes yielded higher diffusion coefficients

as ionic strength was increased. At this point it is not clear if the observed trend is representative of all the lipids in the bilayer or only the fluorescent probe molecules. Similar experiments using a zwitterionic fluorescent probe molecule will address this question.

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